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We are investigating DNA repair as a cellular defense against SM toxicity, seeking conditions that will enhance its effectiveness. One repair mechanism, nucleotide excision repair, provides significant protection against toxicity in cultured Chinese hamster ovary cells. Base excision repair by formamidopyrimidine-DNA glycosylase is also effective *in vitro*, and based on the literature, should be effective *in vivo*. Human alkylpurine glycosylases are also being cloned and tested for activity. Previous studies have shown that mild hypothermia provides protection against cytotoxicity; we believe this is because hypothermia slows progression through the cell cycle and allows more time for repair to occur before cell division takes place. We have found that hypothermia-induced cell cycle arrest is general in nature, involving both G_1 and G_2 arrest, as well as a decreased rate of DNA synthesis in S. Hypothermia-induced cell cycle arrest is accompanied by an increase in levels of the protein factors, p21 and p53 and is dependent on the presence of functional p53.

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David B Ludlum 20 Feb 98

PI - Signature Date

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INTRODUCTION

Background

Sulfur mustard (SM) was introduced as a chemical warfare agent in World War I and is still considered a threat, especially since no effective antidote is available. SM toxicity is unquestionably life-threatening; the compound is acutely toxic to the skin, respiratory tract, and other mucous membranes (1), and is considered carcinogenic by the International Agency for Research on Cancer (2). Our goal is to minimize the toxic effects of SM exposure, primarily by promoting the intracellular repair of SM-induced DNA damage.

Early investigators showed that SM reacts with DNA, and it is generally believed that DNA modification is the initiating event in SM toxicity (3). Related compounds, the nitrogen mustards and other alkylating agents, are widely used in cancer chemotherapy and have antitumor activity that is based on similar reactions with DNA. These therapeutic agents have been widely studied and a great deal of background information is available on the DNA modifications that they produce, and on the ability of cells to protect themselves from this damage (4-6).

DNA damaging agents are not solely the products of the chemical industry; they also occur in nature. For example, an important cellular metabolite, S-adenosylmethionine, is in reality a one-armed sulfur mustard (7,8). Because of the natural occurrence of such compounds, cells have developed defenses against DNA damage in the form of DNA repair enzymes. These enzymes are known to protect cells against the DNA damage inflicted by therapeutic agents, and can presumably provide protection against SM as well (4,6,9). A major objective of our work is to enhance these natural defenses.

Overall, we are investigating the hypotheses that (a) DNA damage is the initiating event in sulfur mustard toxicity, (b) cellular repair of this damage can reduce cytotoxicity, and (c) slowing cell cycle progression through the use of mild hypothermia may allow increased time for repair, thereby decreasing cytotoxicity.

In this Introduction, we have provided a brief review of the DNA damage that is caused by SM and of the cellular enzymes that can repair this damage. The bulk of this report summarizes our progress in the three areas mentioned in the paragraph above.

Sulfur Mustard-induced DNA Modifications

As originally reported by Brooks and Lawley, the principal DNA modification caused by SM is 7-hydroxyethylthioethylguanine (HETEG) which accounts for approximately 60% of the total alkylation (10). This finding has been confirmed repeatedly, most recently by van der Schans *et al.* (11) and by our laboratory (12). Smaller amounts of 3-hydroxyethylthioethyladenine (HETEA) and the cross-link, di-(2-guanin-7-yl-ethyl)sulfide, have also been found consistently. The latter modification is thought to be cytotoxic because it can form interstrand cross-links in DNA.

Studies of chemotherapeutic agents have shown that other DNA modifications that occur infrequently may also be responsible for cytotoxicity. For example, an initial attack at the O^6-position of guanine is responsible for the cyto-

toxic action of the DNA-reactive chloroethylnitrosoureas even though this reaction accounts for only two or three percent of total DNA alkylation (6). By analogy, minor modifications caused by SM cannot be dismissed as unimportant, and several of these including O^6-ethylthioethylguanine have been identified in reactions of the one-armed mustard, chloroethylethyl sulfide, with DNA (13). Similar minor products are probably formed by SM as well, but attempts to identify O^6-hydroxyethylthioethylguanine in DNA that has been reacted with SM have not been successful (14).

Of the various DNA modifications that have been described, however, it seems probable that cross-linking reactions are the most important because bifunctional agents that cause these reactions are much more toxic than monofunctional agents that do not form cross-links (3). Cross-links can be formed within one strand of DNA (intrastrand cross-links), between two strands of DNA (interstrand cross-links), or between DNA and protein. DNA interstrand cross-links are considered particularly significant because they would presumably interfere with DNA replication, but DNA-protein cross-links would probably affect transcription and could also be important causes of cytotoxicity. Evidence that DNA-protein cross-links are indeed formed by SM is included in the Results section.

Repair of SM-induced DNA Modifications

DNA repair can occur directly in a one-step reaction, or more frequently as the result of a series of reactions. The action of O^6-alkylguanine-DNA alkyltransferase illustrates a one-step reaction - the enzyme simply removes an alkyl group attached to the O^6-position of guanine and restores the DNA to its original condition. This enzyme has been particularly well studied because it protects cells from the cytotoxic action of the nitrosoureas, a class of agents used in cancer chemotherapy (15). However, it does not repair the DNA adduct, O^6-ethylthioethylguanine that is formed by the one-armed mustard, chloroethyl ethyl sulfide, and it probably has no role in preventing SM toxicity (13).

Other DNA repair mechanisms recognize and remove a single modified base in a process referred to as base excision repair. Alternatively, an entire oligonucleotide that contains the modified base may be removed by nucleotide excision repair. Additional enzymatic steps are required after these excision reactions to fill in the gaps that remain after the initial excision takes place.

Early investigators obtained evidence for the enzymatic removal of modified bases from the DNA of *E. coli* exposed to low levels of SM (16-18). Subsequently, other investigators have shown that adducts are also removed from the DNA of mammalian cells exposed to SM (19-21).

Papirmeister *et al.* (22) found that bacterial extracts catalyze the release of 3-substituted adenines produced in DNA by 2-chloroethyl-2-hydroxyethyl sulfide (hemisulfur mustard) and attributed this release to the presence of a glycosylase. Although the extracts tested by Papirmeister *et al.* (22) were apparently inactive in releasing the 7-substituted guanine formed by hemisulfur mustard, Habraken *et al.* (23) showed that cloned bacterial 3-methyladenine DNA glycosylase II releases two monoadducts formed by chloroethylethyl sulfide (CEES), 3-ethylthioethyladenine and 7-ethylthioethylguanine. In fact, the activity of this bacterial enzyme towards SM-modified bases was comparable to its activity towards methylated bases.

The activity of human glycosylases towards SM-modified substrates is less clear. Although we have observed activity of partially purified human alkylpurine glycosylase towards this substrate, studies reported herein show that a cloned and purified alkylpurine human glycosylase appears to have no activity. However, recent reports indicate that other mammalian alkylpurine glycosylases exist (24,25), and we will investigate their activity towards SM-modified DNA as they become available to us.

Another bacterial glycosylase, formamidopyrimidine-DNA glycosylase (Fpg protein), is known to provide protection against the toxicity of alkylating agents (26). As described in the Results section, we have shown that this enzyme releases the ring-opened form of the major SM-induced DNA modification, 7-hydroxyethylthioethylguanine. By analogy, the corresponding mammalian enzyme may also provide protection against SM toxicity.

The protective effect of mammalian nucleotide excision repair on SM toxicity is clear and demonstrable as described in the Results section. This repair modality probably has a major role in protecting mammalian cells from SM toxicity.

In summary, the protective action of DNA repair mechanisms has been established, and details of the process are currently being investigated. If hypothermia allows more time for DNA repair to occur, we would expect SM toxicity to be decreased through the use of this modality.

Hypothermia as a Means of Inducing Reversible Cell Cycle Arrest

Important background information on the regulation of the cell cycle has become available from studies of cancer chemotherapy. In that area, if a cell cannot arrest its cell cycle progression to allow time for more DNA repair, the cytotoxicity of a DNA modifying antitumor agent is increased, which is a desirable outcome in a therapeutic setting. Cell cycle progression as it relates to chemotherapy has been reviewed recently with a particular emphasis on the role of p53, a protein that appears to be required for cell cycle arrest (27-32).

Data reported previously show that a period of hypothermia causes a generalized cell cycle arrest that results in improved recovery of cell growth under certain conditions (33). Hypothermia probably slows all metabolic processes including repair, but overall repair of radiation-induced DNA damage has been shown to be increased by a period of hypothermia (34,35). Conversely, hyperthermia increases cell damage by cancer chemotherapeutic agents (36). Thus, we believe that it will be possible to find hypothermic conditions under which DNA repair can be enhanced by modulating cell cycle progression.

In this report, the mechanism of hypothermia-induced cell cycle arrest has been investigated in detail. The data indicate that hypothermia-induced cell cycle arrest, like the cell cycle arrest that follows DNA damage, is mediated by the protein factor, p53.

(continued)

MATERIALS AND METHODS

Materials

Reagents

^{14}C-SM, uniformly labeled in the chloroethyl group, and unlabeled SM were supplied by the Analytical Chemistry Branch, U.S. Army Medical Research In stitute of Chemical Defense. Substrates for *in vitro* repair studies were synthesized from ^{14}C-SM that had a specific activity (SA) = 64.5 mCi/mmol. 2-Chloroethyl-2-hydroxyethyl sulfide (hemisulfur mustard, HSM) was prepared by reacting 2-mercaptoethanol with 1,2-dichloroethane according to Tsou *et al* (37) as described previously (38). Mercaptoethanol, micrococcal nuclease, P1 nuclease, proteinase K, calf thymus DNA, L-cysteine, and all nucleosides and nucleotides were purchased from Sigma. 1-Iodobutane, triethylamine, 1,2-dichloroethane, and sodium hydride were obtained from Aldrich; spleen phosphodiesterase, from Worthington Biochemical Corporation; and T4 polynucleotide kinase, from New England Biolabs. γ-^{32}P-ATP (3000 Ci/mmol) and ^{3}H-thymidine (20 Ci/mmol) were purchased from New England Nuclear. Media and supplements were obtained from Gibco. Chromatography-grade solvents and other reagents were obtained from standard sources.

DNA Repair Enzymes

A plasmid containing the cloned gene for human alkylpurine glycosylase was obtained from Prof. Leona Samson, Harvard School of Public Health, Boston, MA. A new plasmid containing the gene for a histidine-tagged human alkylpurine glycosylase was constructed from this as described in the Results section. The bacterial glycosylase, formamidopyrimidine-DNA glycosylase (Fpg protein), was supplied by our collaborator, Dr. Jacques Laval, of the Institut Gustave Roussy, Villejuif, France. It was isolated and purified as described previously from an over-producing strain of *E. coli* that harbors the pFPG236 plasmid (39).

Cell Lines

Human fibroblasts (AG01522B), obtained from the Coriell Institute for Medical Research, Camden, NJ were at a passage number of 6 and a population doubling level of 15. Mouse embryonic fibroblast Mdm1 cells, which are wild type for p53, and A3 cells, which are p53 null mutants, were obtained from Dr. Stephen N. Jones, Department of Molecular Genetics and Microbiology, UMass Medical Center; both cell lines were at passage 2 (40,41). Chinese hamster ovary (CHO) cell lines that are competent (AA8, wild type) or deficient (UV41) in nucleotide excision repair were obtained from the American Type Culture Collection.

Methods

HPLC Analysis

HPLC analyses and isolations of synthetic products were performed on a modular HPLC apparatus consisting of a Milton Roy minipump, a Rheodyne 7125 in jector valve, and 5 μm Spherisorb (4.6 x 250 mm) C_{18} columns. The ultraviolet absorbance of the eluent was monitored by a Hewlett Packard 1040A diode array detector interfaced with a Hewlett Packard 85B computer with hard disk drive which recorded spectra of derivative peaks as they appeared. When radioactiv-

ity was involved, 1 min fractions were collected in minivials, 3.5 ml of Hydrofluor was added to each vial, and fractions were counted in an LS-6500 Beckman scintillation counter. Columns were generally eluted with 25 mM KH_2PO_4, pH 5.5 buffer, and a methanol or acetonitrile gradient as described (33,42).

Synthesis of HPLC Markers

SM-modified nucleobases and nucleotides were synthesized as described previously (33). Ring-opened 7-hydroxyethylthioethylguanine (ro-HETEG) was prepared by treating 7-hydroxyethylthioethyldeoxyguanosine 5'-phosphate with 2 M NH_4OH for 20 h under nitrogen at 37°C. The ring-opened product was concentrated by lyophilization and dissolved in 75% formic acid for depurination. This solution was incubated for 4 h under nitrogen at 37°C and separated by HPLC on a C_{18} column. The depurinated product appeared as two poorly resolved peaks with similar UV scans that were evidently the two rotamers of ro-HETEG (43). These two peaks were collected separately, desalted on a C_{18} column using water as an eluent, and characterized by ultraviolet and mass spectrometry. Ring-opened 7-methylguanine was prepared and purified from 7-methylguanosine by a similar method.

Synthesis of a Putative DNA-protein Cross-link

Since the most reactive sites in DNA and proteins are the N-7 position of guanine and the sulfhydryl group of cysteine, respectively, we believe the structure shown in Figure 1 below may link the two molecules together. Our synthetic route to this structure is shown in Figure 2 (next page).

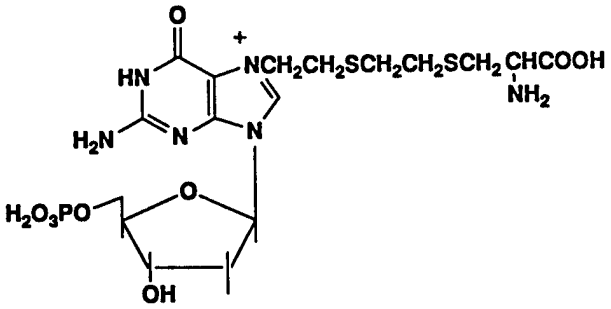


Figure 1. Proposed Structure of a DNA-protein Cross-link. This entity would remain after complete nuclease and protease digestion of a nuclear protein cross-linked to DNA through the N-7 position of guanine.

The reaction scheme shown in Figure 2 was performed initially as a "one-pot" synthesis; the intermediate products were not isolated. L-Cysteine (5 mg, 40 μmol) and $NaHCO_3$ (5 mg, 6 μmol) were dissolved in 70 μl of H_2O in a 5 ml round bottom flask and hemisulfur mustard (4.4 μl, 40 μmol) was added with stirring under nitrogen. After 4 h of reaction at room temperature, the solution was taken to dryness, concentrated HCl (1 ml) was added, and the solution was heated under reflux to 90°C for 6 h. The solution was again taken to dryness and a sample of the residue was tested for its ability to alkylate nitrobenzyl pyridine; alkylation occurred when the solution was heated to 100°C for 20 min. Accordingly, deoxyguanosine 5'-phosphate (7.3 mg, 20 μmol) dissolved in 1 ml of 0.1 M sodium cacodylate buffer was added to the residue, and the solution was heated to 100°C for 20 min. The complex reaction mixture was separated by HPLC

and individual peaks were isolated for ultraviolet and mass spectral analysis.

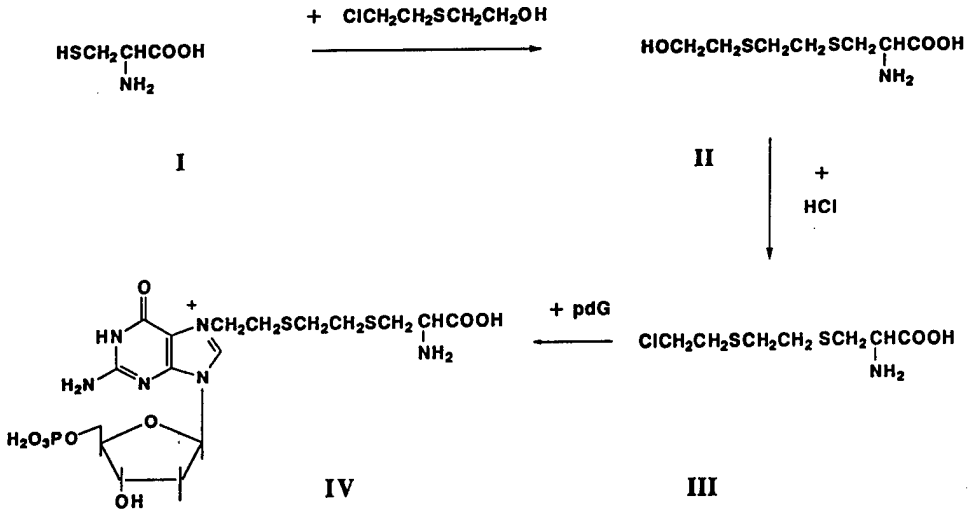


Figure 2. Synthetic Route to the Proposed DNA-protein Cross-link.

Molecular Spectroscopy

Ultraviolet spectra were obtained on a Hewlett Packard 8452A diode array spectrometer. Mass spectrometry was performed by Mr. Marion C. Kirk at the University of Alabama (Birmingham, AL) on a Varian MAT 311A instrument using a sulfolane-glycerol matrix and an 8 keV beam of xenon atoms.

DNA Repair Studies

Experiments with DNA Glycosylases

Preparation of Substrates

Substrates for glycosylase assays were prepared as described (42,44). Briefly, calf thymus DNA was alkylated with either ^{3}H-methylnitrosourea (^{3}H-MNU-DNA) or ^{14}C-sulfur mustard (^{14}C-SM-DNA), washed free of unbound radioactivity, and characterized for modified base content by acid hydrolysis and HPLC analysis (42,44). All operations involving SM were carried out in a special SterilchemGARD hood (Baker Company Inc., Sanford, ME) with bag in/bag out charcoal filters; personnel were protected with double vinyl gloves and laboratory coats. Substrates containing the ring-opened forms of 7-methylguanine or 7-hydroxyethylthioethylguanine were prepared from the methylated or SM-treated substrates by exposure to alkaline conditions (42). Again, substrate composition was determined by acid hydrolysis and HPLC analysis.

Isolation of Enzymes

Bacterial 3-methyladenine DNA glycosylase II was isolated from *E. coli* harboring the plasmid pYN1000 as described previously (12). Purification of cloned human enzyme from the plasmid obtained from Professor Samson proved to be difficult, and a new plasmid was constructed as described in the Results section that contains the gene for the human enzyme with a his_6 tag

at the carboxyl end. This plasmid was grown in *E. coli* and enzyme was isolated from it on a Ni-Agarose column as described in the Results section.

Glycosylase Assays

Glycosylase assays followed the procedures described in references 42 and 44. Briefly, substrate containing approximately 20,000 cpm of modified bases was incubated at 37°C in pH 7.5 assay buffer for 10 to 30 min in the presence of varying amounts of glycosylase enzyme. At the end of the incubation, substrate and enzyme were precipitated with ethanol, and the mixture was centrifuged. Centrifugation leaves the bases that are released from the substrate by the glycosylase in the supernatant; the extent of the release was determined by counting an aliquot of the supernatant in a scintillation counter.

Effect of Nucleotide Repair on Viability of SM-Exposed CHO Cells

Wild type cells and those lacking in nucleotide excision repair were exposed for one hour at room temperature to varying concentrations of SM freshly diluted in medium; at the end of this time, the medium containing SM was replaced with fresh medium, and incubation was continued at 37°C. Since CHO cells grow both as a monolayer and in suspension, the medium was collected before trypsinization and cells from both the medium and the monolayer were combined for analysis at the indicated times. Cells were trypsinized, washed and resuspended in PBS and tested for trypan blue exclusion to assay for cell viability as described (33). Analysis of duplicates showed that these counts were reproducible with a standard deviation of ± 6.3%.

Cell Culture Studies

Growth Conditions

Human fibroblasts (AG01522B) were grown as a monolayer in standard minimal essential media (MEM) with a 2 x concentration of amino acids and vitamins supplemented with 15% fetal bovine serum (FBS), penicillin and streptomycin. Cells were incubated in a humidified atmosphere of 7% CO_2 at 37°C. Chinese hamster ovary (CHO) cells were grown in a humidified atmosphere of 7% CO_2 at 37°C in Alpha minimum essential medium (Eagle) without ribonucleosides or deoxyribonucleosides, but supplemented with 10% FBS. Mouse embryonic fibroblasts were grown at either 37°C or 28°C in standard DMEM medium supplemented with 15% fetal bovine serum and antibiotics. Incubation temperatures in these experiments were monitored with a Yellow Springs Instrument Model 4600 Digital Thermometer.

Cell Synchronization

Normal human fibroblasts were synchronized in G_0 by serum starvation as described (45). Exponentially growing cells were washed three times with serum-free media, and then incubated at 37°C in fresh media containing 0.5% serum. After 48 h, the media was replaced with fresh low-serum media and the cells were incubated for an additional 24 h. At this point, the cells were synchronized in G_0, and fresh growth media containing 15% serum was added for experiments on the effects of hypothermia.

Cells were arrested at the G_1/S boundary through the use of aphidicolin. Fibroblasts were plated at a density of 2 x 10^4 cells/cm^2 and incubated in media containing 15% serum at 37°C. Under these conditions, the cells initiate DNA synthesis about 14 h after plating. Accordingly, 2.5% aphidicolin, which prevents DNA synthesis, was added 7 h after plating and cells were incubated in its presence for 13 h. Aphidicolin was removed by rinsing the cell monolayer three times with media; then, fresh media was added and the synchronized cells were used for experiments on hypothermia.

Viability Analysis

Viability was assayed by the trypan blue exclusion assay. Growing cells were trypsinized, washed and resuspended in PBS. After staining, trypan blue-negative (excluding) cells were counted in a hemocytometer. Analysis of duplicate determinations shows that these counts are reproducible with a standard deviation of ± 6.3%.

Cell Cycle Analysis

Cell cycle distribution was determined as DNA content per cell by propidium iodide (PI) staining. Approximately 1 x 10^6 cells were fixed with ethanol and kept at 4°C for at least 24 h before analysis. Prior to staining, low molecular weight DNA was extracted by the procedure described by Hotz et al. (46). This extraction allows identification of cell populations with fractional, sub-G_1 DNA content that is indicative of DNA fragmentation and cell death. Ethanol-fixed cells were pelleted and incubated for 30 min in pH 7.8 buffer containing 50 mM Na_2HPO_4, 25 mM citric acid (9:1) and 0.1 % Triton X-100. Cells were then stained for 30 min at room temperature in 10 mM piperazine-N,N'-bis(2-ethanosulfonic acid) (PIPES) buffer (pH 6.8) containing 0.1 N NaCl, 2 mM $MgCl_2$, 0.1 % Triton X-100, 20 μg/ml PI and 50 μg/ml RNase. Cell cycle analysis was performed using a Becton-Dickinson FACScan flow cytometer (Mountain View, CA). At least 15,000 events were collected per sample; cell doublets and aggregates were electronically eliminated from analysis. The percentage of cells in each phase of the cell cycle was determined using Modfit software (Verity Software House, Topsham, ME).

^{3}H-Thymidine Incorporation

DNA synthesis has been followed by ^{3}H-thymidine incorporation. Briefly, fibroblasts were grown in monolayers in 2 ml of media in 35 mm petri dishes. At the indicated time, cells were incubated for 30 min in the presence of ^{3}H-thymidine at a concentration of 1 μCi/ml of media. The media containing radioactivity was then removed and the cells were washed with 2 ml of ice-cold PBS. Two ml of trichloroacetic acid (TCA, 10%) was added, cells were put on ice for 5 min, TCA was aspirated and replaced with 2 ml of fresh TCA, and the cells were put on ice for an additional 5 min. TCA was then aspirated and 0.5 ml of SDS (10%) was added at room temperature; a minimum of 2 min was allowed for the TCA precipitates to dissolve. The cell layer was scraped into a scintillation vial and the well was rinsed with additional 0.5 ml SDS which was added to the same vial. UltimaGold scintillation fluid from Packard (2.5 ml) was added and radioactivity was measured in a Beckman LS 6500 scintillation counter.

Western Blot Analysis

For Western blot analysis, cells were harvested by trypsinization at either 37°C (for cells grown at 37°C) or at 28°C (for cells grown at 28°C), rinsed with PBS and stored at -20°C until analysis. Samples were lysed on ice for 30 min in 50 mM Tris-HCl buffer, pH 7.5, containing 150 mM NaCl, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 100 KIU/ml aprotinin, and 10 μg/ml leupeptin. Cell lysates were centrifuged for 10 min at 12,000 rpm, and the protein concentration in each supernatant was determined by the BioRad protein assay. Samples were boiled for 5 min in a loading buffer of 50 mM Tris-HCl, pH 6.8, containing 10% glycerol, 2% sodium dodecyl sulfate (SDS), 0.1% bromphenol blue and 28 mM (β-mercaptoethanol. Proteins (50 μg/lane) were resolved by electrophoresis in 10 % polyacrylamide-SDS gels and transferred to a polyvinylidene fluoride membrane (BioRad, Hercules, CA) with a transfer buffer of 25 mM Tris-HCl containing 192 mM glycine and 10 % v/v methanol. Biotinylated protein marker (New England Biolabs, Beverly, MA) was used as a molecular weight standard. After transfer, the gel was stained with Coomassie blue as a control for loading and transfer. The membrane was probed with the p53-specific monoclonal antibody, Pab1800 (Oncogene Research Product - Calbiochem, Cambridge, MA) and then reprobed with p21-specific antibody WAF1 (Ab-1) (Oncogene) and with monoclonal anti-β-actin N350 antibody (Amersham Life Science, Arlington Heights, IL). Immunoreactive proteins were detected with alkaline phosphatase-conjugated anti-mouse immunoglobulin (IgG) and a chemiluminescent reagent (CDP-star) (New England BioLabs). After autoradiography, signals were quantitated by densitometric scanning with a Molecular Dynamics Personal Densitometer. Analysis was performed using the ImageQuant (version 1.1) program. Levels of p53 and p21 proteins were determined relative to the levels of β-actin.

RESULTS

Formation of DNA-protein Cross-links by SM

When DNA is isolated from cells that have been exposed to SM and subjected to the ^{32}P-postlabeling technique, HPLC profiles of the labeled nucleotides show a new peak of radioactivity that is not present in DNA that has been exposed to SM *in vitro* (47). A typical example of this is shown in Figure 3 (next page). Based on the specificity of the labeling and isolation methods, this new peak is an N-substituted deoxyguanosine 5'-phosphate. Since it has a different retention time from HETEpdG and the corresponding sulfoxide and sulfone, we believe it is the residue of a DNA-protein cross-link as explained in the Introduction.

The products of the "one pot" synthesis that was undertaken to produce such a cross-link *in vitro*, as described under Methods, were separated by HPLC under similar conditions, but with a milder gradient, than was used in Figure 3; this profile is shown in Figure 4. Besides the reactant deoxyguanosine 5'-phosphate (pdG) and its depurination product guanine (G), several new products were observed. The two largest peaks, marked "unknown I" and "unknown II", both had ultraviolet spectra consistent with an N-7 substituted deoxyguanosine 5'-phosphate. These two products were analyzed by mass spectometry and found to have formula weights of 495 and 452, respectively. These formula weights would correspond to structures I and II in Figure 5, rather than the desired product shown in Figure 1. Accordingly, the synthesis is being repeated with intermediate purification steps as described in the Discussion.

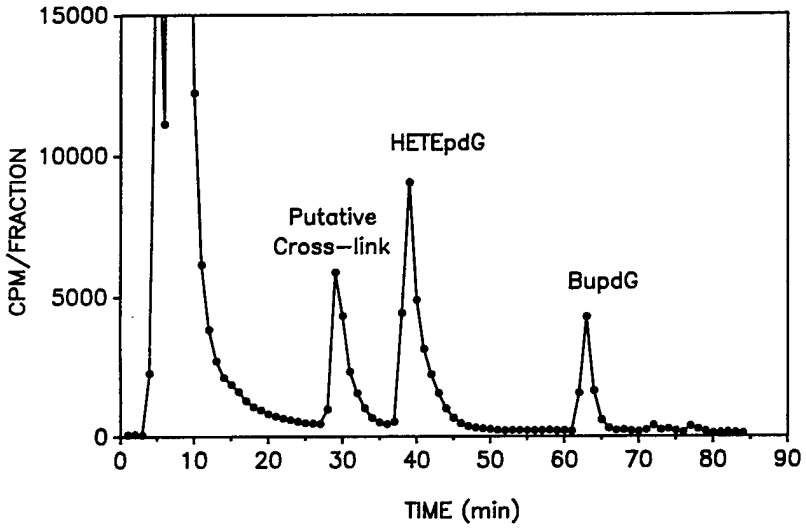


Figure 3. HPLC Profile of ^{32}P-postlabeled Nucleotides from DNA of Human Fibroblasts Exposed to SM in Cell Culture. DNA, isolated from fibroblasts that had been exposed to 100 μM SM for 1 h, was digested and subjected to postlabeling as described in reference 47. Labeled nucleotides were separated by HPLC on a C_{18} column; 1 min fractions were collected and counted. A peak labeled "putative cross-link" eluted in front of the principal DNA adduct, HETEpdGp, and the internal standard peak, BupdG.

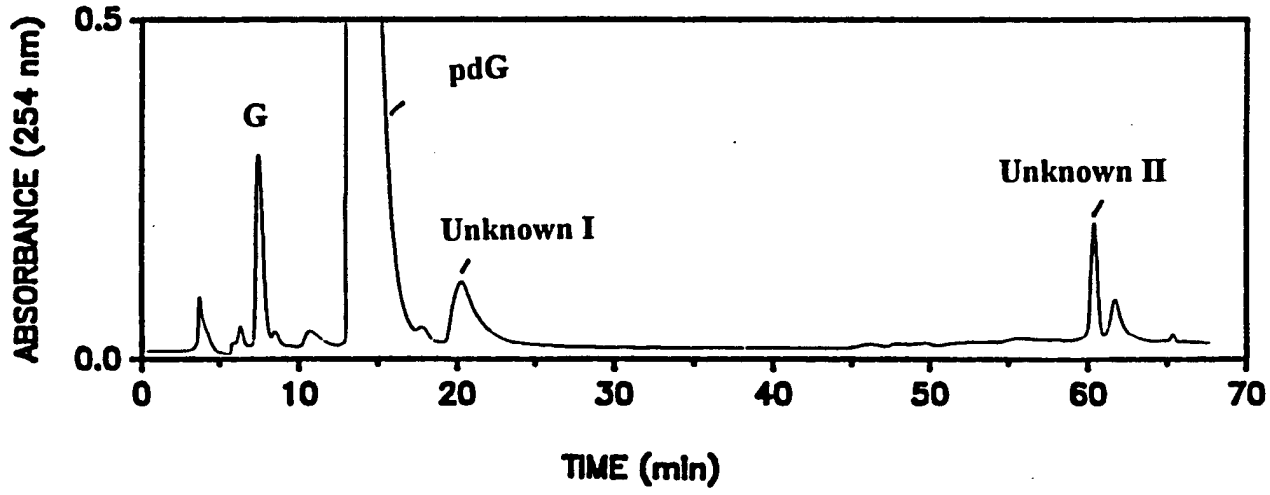
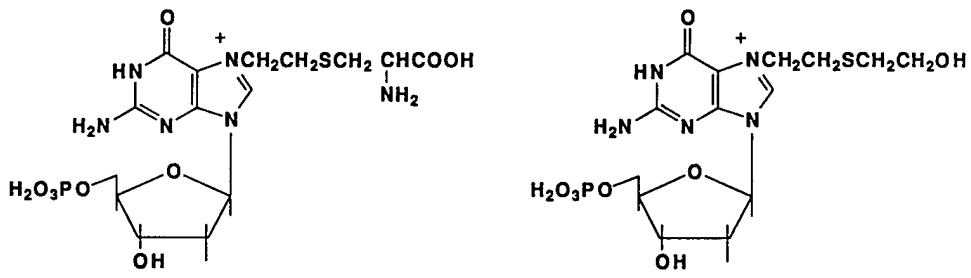


Figure 4. HPLC Profile of Products Obtained in the Reaction Shown in Figure 2. A sample of the reaction products was eluted from a C_{18} column using a slower gradient than that used in Figure 3.



I II

Figure 5. Structures of Products Isolated from the Reaction Shown in Figure 2.

Repair of SM-induced DNA Modifications

Action of Glycosylases on SM-modified DNA

Cloned Human Alkylpurine Glycosylase

Previous investigations have shown that bacterial alkylpurine glycosylase (specifically, 3-methyladenine DNA glycosylase II) releases SM-modified bases from DNA (44). Recent studies show that a corresponding mouse enzyme protects against the cytotoxicity of several alkylating agents (48). Consequently, we have had an intense interest in testing the activity of human alkylpurine glycosylase against SM-modified DNA.

Although we obtained a plasmid containing a cloned human alkylpurine glycosylase from Professor Leona Samson of the Harvard School of Public Health, we obtained poor yields of the enzyme and purification was difficult. We have been fortunate in obtaining the collaborative help of Dr. Michael R. Volkert of the University of Massachusetts Department of Molecular Genetics and Microbiology in constructing a plasmid that expresses a histidine-tagged human glycosylase. The construct started with plasmid pBU16, a gift from Professor Samson. Plasmid pBU16 carries a Bsu36I-XbaI from pKT218 that encodes the human alkyladenine DNA glycosylase gene (hAAG) in the pSL301 vector (Invitrogen Corp., San Diego, CA) (49). (Professor Samson refers to this enzyme as alkyladenine glycosylase rather than alkylpurine glycosylase, but we prefer the latter designation because other investigators have shown that human glycosylases can also act on alkylguanine bases.)

We subcloned the hAAG-bearing EcoRI-HinDIII fragment from pBU16 into pTrc99A (Pharmacia Biotech) to produce plasmid pMV503. The pTrc99A vector carries the lacI gene of *E. coli* and has the synthetic trc promoter under lac operator control positioned upstream of the EcoRI site. The presence of lacI on the plasmid results in tight repression of the promoter which can be induced by the addition of the lac inducer isopropyl β-D-thiogalactopyranoside (IPTG), and thus allows IPTG inducible expression of hAAG. The presence of the wild type

hAAG gene on pMV503 was confirmed by restriction digestion with BstEII, CelII, BglI, EcoRI, HinDIII and SacI followed by gel electrophoresis.

Plasmid pBU16 produces a fusion protein of hAAG and vector-encoded sequences 5′ to hAAG. To remove the pBU16 vector coding sequences from the 5′ end of the hAAG gene the EcoRI-BstEII fragment of pMV503 was removed by first digesting to completion with EcoRI, then partial digestion with BstEII to produce a 5152 bp fragment. This fragment was purified from a 0.8% agarose gel using the Gene Clean System (Bio101, Inc). Oligonucleotides MV1, (AATTCTAAGGAGGTATCTAATG), and MV2, (GTGACCATTAGATACCTCCTTAG), were first annealed to one another by heating and slow cooling, then ligated to the purified 5152 bp EcoRI-BstEII fragment of pMV503 to produce plasmid pMV509. These oligonucleotides have four important features: (1) they are complementary to one another, (2) when annealed, they produce single stranded ends complementary to the EcoRI and BstEII sites of pMV503, (3) they reconstruct the ATG initiation codon of hAAG, which lies within the BstEII site, and (4) they contain a consensus ribosome binding site, AGGAGG, appropriately positioned to allow optimal translation initiation from the downstream ATG initiation codon. The resulting plasmid, pMV509, carries the wild type hAAG under the control of the IPTG inducible P*trc* promoter of pTrc99A. Construction of pMV509 was confirmed by loss of the SalI restriction site that lies between EcoRI and BstEII of pMV503 and restoration of the EcoRI and BstEII sites.

To construct the histidine tagged hAAG gene, pMV509 was digested to completion with CelII and HinDIII and the 5064 bp fragment was purified from a 0.8% agarose gel as described above to remove the 3′ end of the gene. The oligonucleotides MV3 (TGAGCAGGACACACAGGCCCATCATCATCATCATCACTGA) and MV4 (AGCTTCAGTGATGATGATGATGATGGGCCTGTGTGTCCTGC) were first annealed to one another as described above, then ligated to the 5064 bp fragment of pMV509 to produce plasmid pMV513. Oligonucleotides MV3 and MV4 have four important features: (1) they are complementary to one another, (2) when annealed, they produce single stranded ends complementary to CelII and HinDIII, (3) they restore the last seven amino acid codons of the hAAG gene in the appropriate reading frame, and (4) they add six histidine codons and one stop codon to the 3′ end of the hAAG gene. Construction of pMV513 was confirmed by loss of the MluI restriction site that lies within the CelII-HinDIII fragment of pMV509 and restoration of the CelII and HinDIII sites.

Since the hAAG gene was initially cloned by functional complementation of an *E. coli* mutant strain lacking the *alkA* and *tag* DNA glycosylase genes (49), function of the wild type and histidine tagged hAAG genes was tested by assessing the ability of pMV509 and pMV513 to complement the alkylation sensitivity of the *alkA1 tag-1* double mutant *E. coli* strain MV2157. Both plasmids are able to restore MNNG resistance to MV2157 after IPTG treatment as expected if both the wild type and histidine tagged hAAG genes were inducible and active.

The hAAG-his$_6$ enzyme was actually isolated from strain MV4126 (*alkA1tag-1*/pMV513). Cells were grown to a Klett reading of 70 (approximately 5 x 10^8 cells/ml) in LB broth containing ampicillin (100 μg/ml), then induced by the addition of IPTG (2 mM). Fresh ampicillin (100 μg/ml) was also added at this time to insure plasmid maintenance, and cells were incubated for an additional 5 hours.

After incubation cells were centrifuged, washed in saline buffer (10 mM Tris-Cl, pH 7.4; 1 mM EDTA; 100 mM NaCl) and lysed using a Kraft homogenizer

followed by a French press. Crude cell extracts were then centrifuged at 12,000 x g and the supernatant recovered. The supernatant was applied to a Ni-agarose column (Qiagen) equilibrated with column buffer (5 mM Na_2HPO_4, pH 8.0; 300 mM NaCl; 10% glycerol). The column was washed with 200 ml of column buffer followed by 300 ml of 30 mM imidazole in column buffer, and finally eluted with a 100 ml gradient of 30 mM to 500 mM imidazole in column buffer. Two ml fractions were collected and assayed for glycosylase activity with [^{3}]H-MNU-modified DNA. A sharp peak of activity appeared around fraction 26.

As shown in Figure 6 below, the purified enzyme has the expected activity towards a methylated substrate, but no detectable activity towards SM-modified DNA.

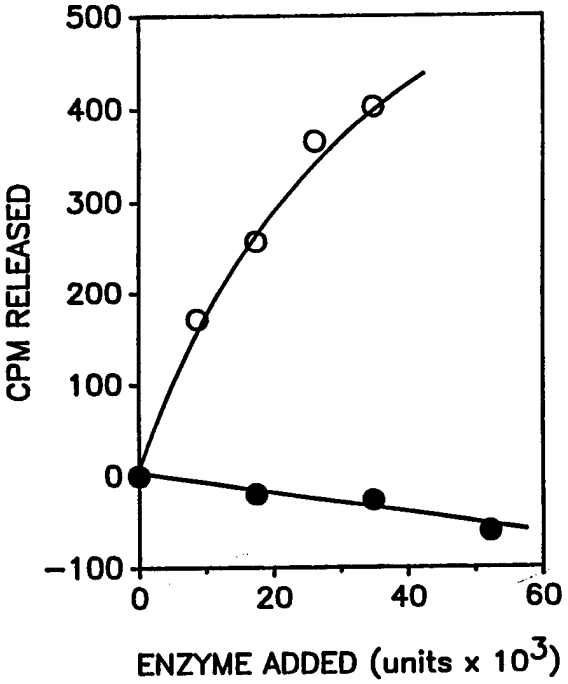


Figure 6. Lack of Release of SM-Modified Bases from DNA by Cloned Human Glycosylase. DNA modified by either ^{3}H-methylnitrosourea (O) or by ^{14}C-SM (●) was incubated with cloned human enzyme at 37°C as described in Methods. DNA and protein were then precipitated and the bases that had been released into the supernatant by glycosylase action were determined by liquid scintillation counting.

There are two possible explanations for this lack of activity: either mammalian cells use other repair mechanisms besides glycosylases to remove SM-modified bases from their DNA, or other mammalian glycosylases besides the one that we have cloned act on SM-modified DNA. The latter explanation seems likely in view of recent data indicating that mammalian cells have more than one glycosylase (24,25).

Formamidopyrimidine-DNA Glycosylase (Fpg Protein)

This bacterial glycosylase has marked activity towards the ring-opened form of 7-hydroxyethylthioethylguanine. An HPLC profile of the bases released by acid hydrolysis from alkaline-treated [^{14}C]SM-DNA is shown in the first panel of Figure 7. Three peaks of radioactivity were released which co-

eluted with optical markers for ro-HETEG, HETEA and HETEG respectively. As shown in the second panel of Figure 7, control incubations with buffer alone released no radioactivity while incubation with Fpg protein released ro-HETEG only.

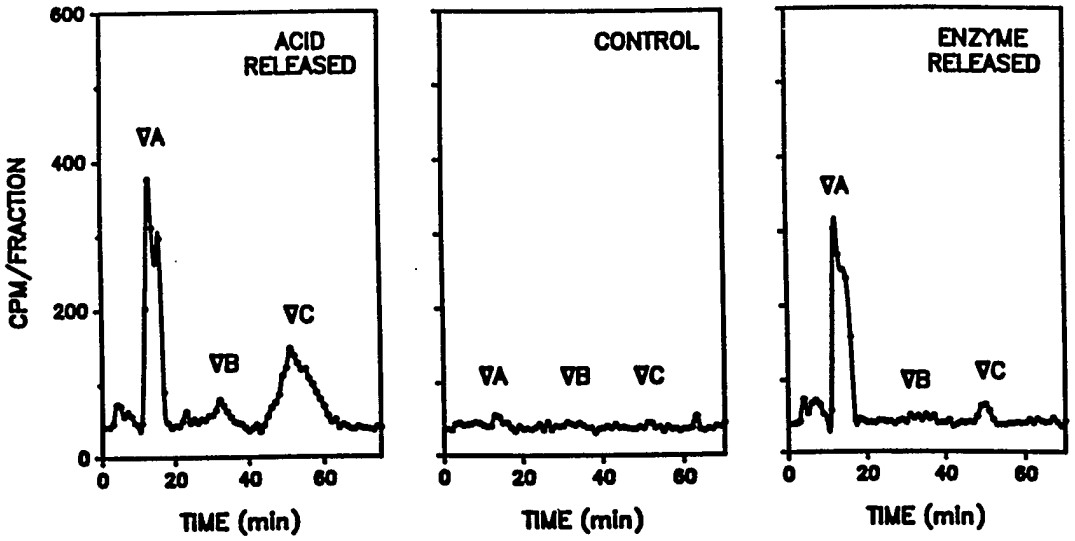


Figure 7. HPLC Profiles of Bases Released from Ring-opened SM-modified DNA. Retention times are indicated for optical markers of: A, ro-HETEG; B, HETEA; and C, HETEG. The panels from left to right show radioactivity released by acid depurination, control incubation with buffer alone, or incubation with Fpg protein for 15 min at 37°C (see text for details).

The enzyme dependence for release of ro-HETEG is shown in Figure 8 (next page). Substrate containing 37.0 pmols of alkylated bases including 17.1 pmols of ro-HETEG was incubated with increasing amounts of enzyme for 15 min at 37°C. As shown in this figure, release of ro-HETEG is clearly enzyme dependent, but appears to level off when approximately 50% of the total pmols of ro-HETEG present in the substrate have been released.

The time dependence curve for release of ro-HETEG shown in the right-hand panel of Figure 8 indicates that release of ro-HETEG occurs rapidly over the first few minutes of incubation. Again, however, the number of pmols of ro-HETEG released levels off when approximately 50% of the total has been reached.

We conclude that the Fpg protein is effective in removing ro-HETEG from SM-damaged DNA. By analogy with the protection that the Fpg protein affords against aziridine toxicity, we believe that it may have a role in protecting cells from SM toxicity.

(continued)

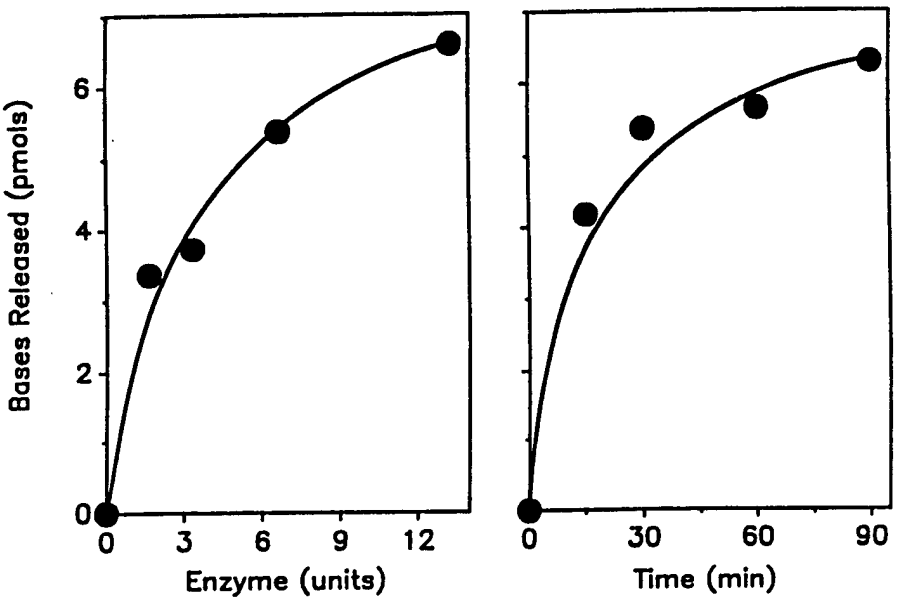


Figure 8. Enzyme- and Time-dependent Release of ro-HETEG by Fpg Protein. Left panel: substrate containing 17.1 pmols of ro-HETEG was incubated with the indicated amounts of Fpg protein in 100 μl buffer for 15 min at 37°C. Right panel: substrate containing 17.1 pmols of ro-HETEG was incubated with 3.3 units of Fpg protein in 100 μl buffer at 37°C for the indicated time periods (see text for details).

Protection against SM Toxicity by the Nucleotide Excision Repair Pathway

In addition to base excision repair, bacterial and mammalian cells possess an alternate nucleotide excision repair pathway that can clip out lengths of DNA that contain a modified base. Since this system is composed of a group of proteins acting in concert, it is difficult to study *in vitro*. However, mutant Chinese Hamster Ovary (CHO) cells are available that lack one or more elements necessary for this repair pathway to operate. Using these cells, it is possible to test the importance of the nucleotide excision repair pathway by comparing the survival of cells that are able to repair DNA in this way with mutant cells that lack this ability.

As shown in Figure 9 (next page), mutant cells are far more sensitive to SM toxicity than wild type cells. Although the wild type and mutant cells grow at a similar rate in the absence of SM (left panel), exposure to 5 μM SM results in a rapid loss of mutant cells whereas wild type cells are retarded, but begin to recover in a few days.

We conclude from this and similar experiments that nucleotide excision repair is important in protecting mammalian cells from SM toxicity.

(continued)

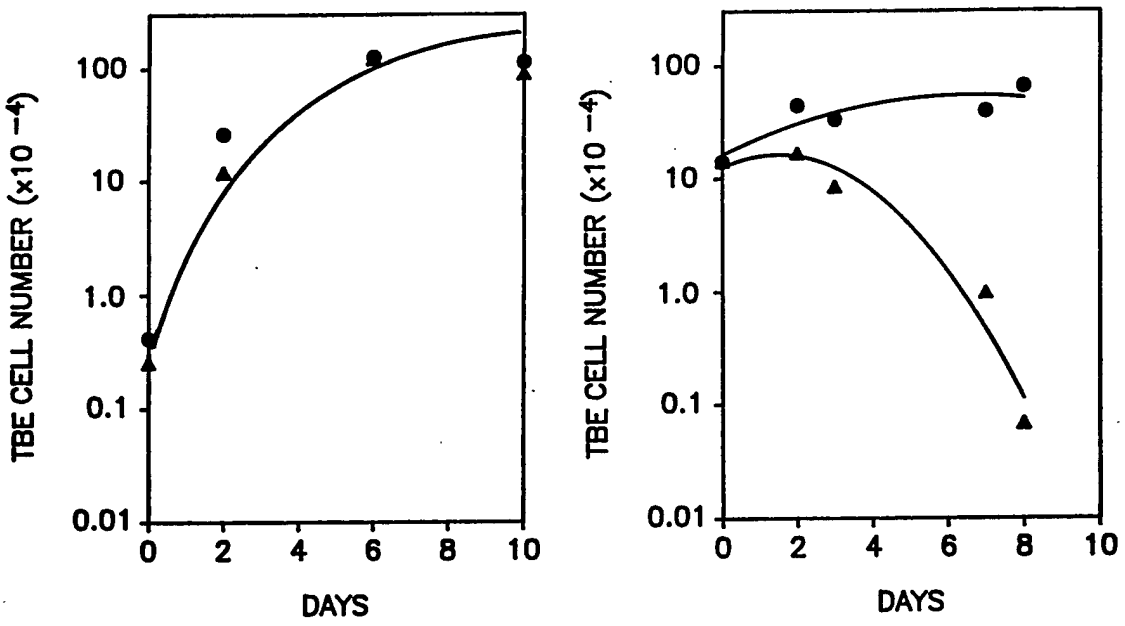


Figure 9. Effect of Nucleotide Excision Repair on SM Toxicity. Growth, measured by the number of trypan blue excluding cells, is plotted *versus* days of incubation. The left panel shows the growth of wild type CHO cells (●) and nucleotide excision repair-deficient CHO cells (▲) under standard conditions, and the right hand panel shows growth after exposure to 5 μM sulfur mustard.

Studies of Hypothermia-induced Cell Cycle Arrest

Pursuing the hypothesis that hypothermia might slow the cell cycle and allow more time for DNA repair after exposure to SM, we began by examining the growth of human fibroblasts at various temperatures. As shown in Figure 10 (next page), cells continued to grow and increase in number at 31°C, but were essentially arrested at 28°C.

The next step was to examine the ability of cells to recover from short periods of hypothermia. Cells were incubated for varying lengths of time at 28°C and then transferred back to 37°C. As shown in Figure 11 (next page), cells that were incubated for as long as four days at 28°C grew at a rate characteristic of 37°C after a brief recovery period. We conclude from this experiment that cells are able to survive fairly long periods of mild hypothermia.

As reported previously (33), cells that had been incubated for one day at 28°C after exposure to SM before being transferred back to 37°C had a higher survival than cells that were incubated at 37°C continuously after exposure. To determine optimum conditions of hypothermia for exposed skin would require studies in a more complex biological system than the cell culture system used in these experiments, but cell culture systems are ideal for examining the nature of the cell cycle arrest and the activity of DNA repair systems under hypothermic conditions.

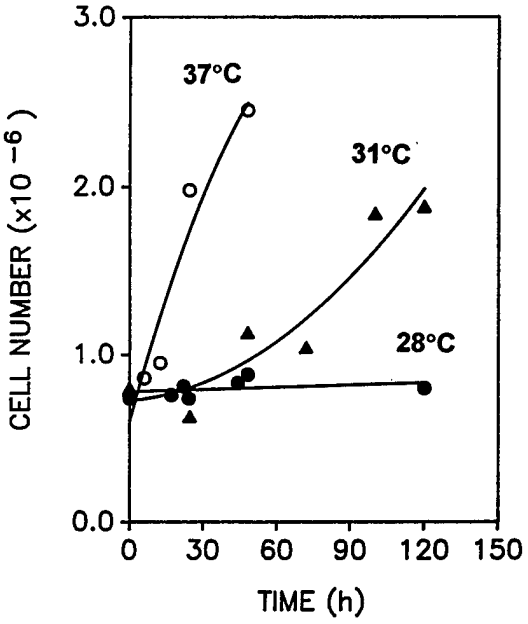


Figure 10. Effect of Hypothermia on Growth of Human Fibroblasts. Cells were plated and grown at 28°C (●), 31°C (▲), or 37°C (○).

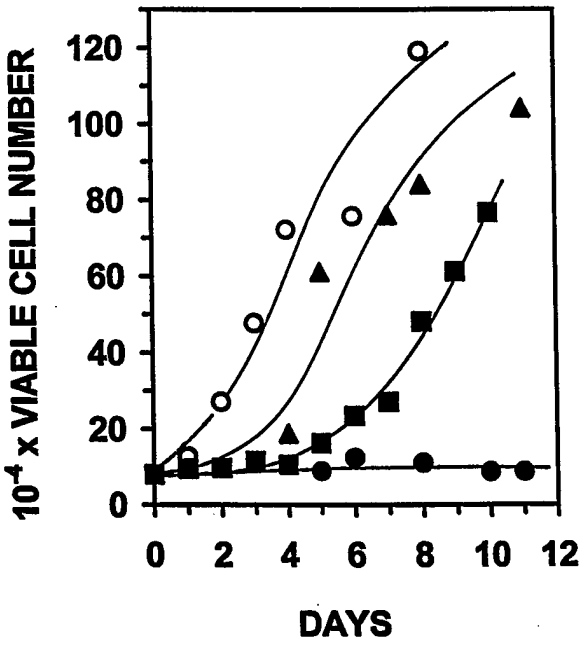


Figure 11. Recovery of population growth at 37°C after hypothermia-induced growth arrest. AG01522 cells were either incubated continuously at 37°C (○) or at 28°C (●), or they were grown at 28°C for two (▲) or four (■) days and then transferred to 37°C. The viable cell number was determined by the trypan blue exclusion assay.

To examine the effects of hypothermia on cell cycle progression, normal human diploid fibroblasts (AG01522) were plated at a density of 2 x 10^4 cells/cm^2 and incubated continuously at 37°C, 31°C, or 28°C. Subsequently, cells were harvested and cell cycle analysis was performed as described above. The top panel of Figure 12 illustrates the cell cycle distribution, measured as DNA content per cell, in populations of AG01522 cells grown at the three temperatures. Progression into the S phase is clearly delayed at the lower temperatures; compared to cells grown at 37°C, progression into S phase is delayed for at least two days at 28°C.

This can be seen quantitatively in the bottom panel of Figure 12 which shows the percent of cells in each phase of the cell cycle as a function of time. After four days, cells at 28°C start to accumulate in G_2/M; by day five, the

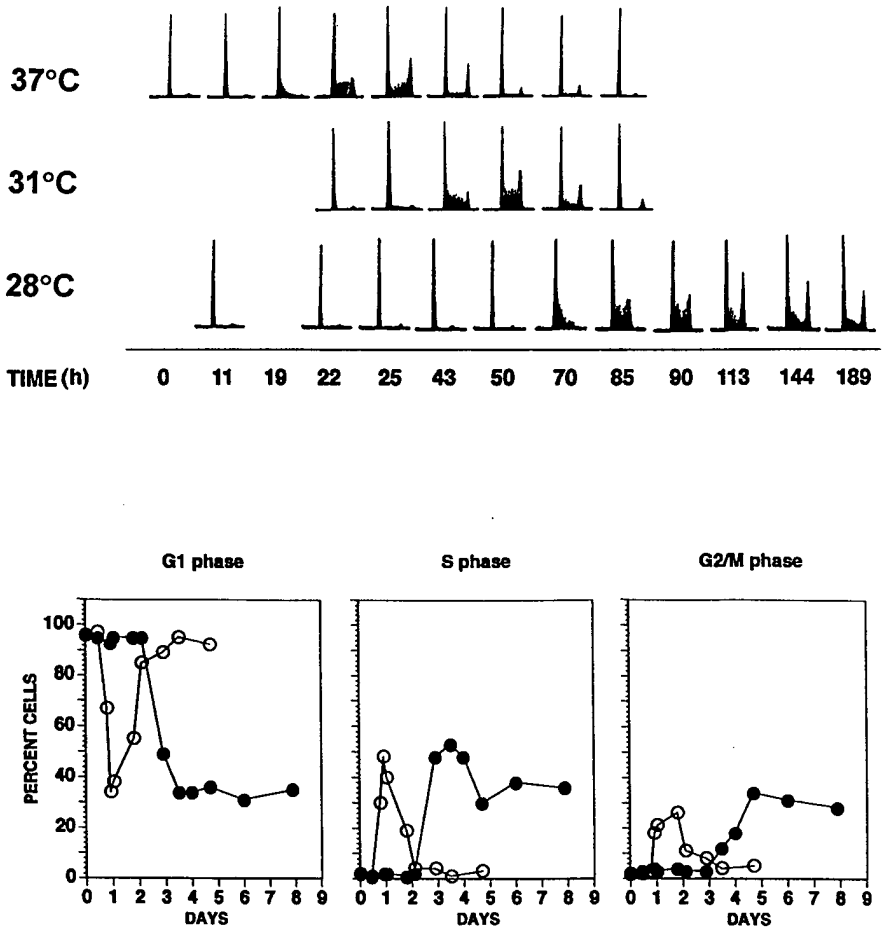


Figure 12. Effect of hypothermia on cell cycle progression in normal human AG01522 fibroblasts. Cells were plated at a density of 2 x 10^4 cells/cm^2 and incubated at either 37°C, 31°C, or 28°C; cell cycle analysis was performed as described in the text. Top panel: DNA histograms at 37°C (top row), at 31°C (middle row), and at 28°C (bottom row). Bottom panel: Percentage of cells in each phase of the cell cycle at 37°C (O) and at 28°C (●).

population is nearly equally distributed among the G_1 (36%), S (30%) and G_2/M (34%) phases. This distribution does not change for at least the next nine days. The data for day 14 (not shown) were: G_1, 25%; S, 43%; and G_2/M, 32%. A lack of increase in the percentage of cells in G_1 for the entire duration of the experiment suggests that cells are not progressing into the next cell cycle. This is in agreement with the data on cell growth as determined by the trypan blue exclusion assay. As shown in Figure 11, there was no increase in the number of viable cells in the population incubated at 28°C for at least 10 days in this experiment. On the other hand, there were no indications of cell death either by trypan blue exclusion criteria or from cell cycle analysis data. This suggests that the constant viable cell number in the population of AG01522 cells at 28°C results from cell cycle arrest rather than from a balance between cell proliferation and cell death.

Since the advantage of hypothermia would be to provide extra time for DNA repair before DNA synthesis and cell division, we have investigated the effects of hypothermia on DNA synthesis.

Using ^{3}H-thymidine labeling, cells synchronized in G_0 and plated at 37°C undergo two waves of DNA synthesis approximately 20 h and 40 h after plating as shown in Figure 13. Cells transferred to 31°C, however, enter the S phase much later, reaching a peak at approximately 45 h as shown in Panel A of Figure 14 (next page).

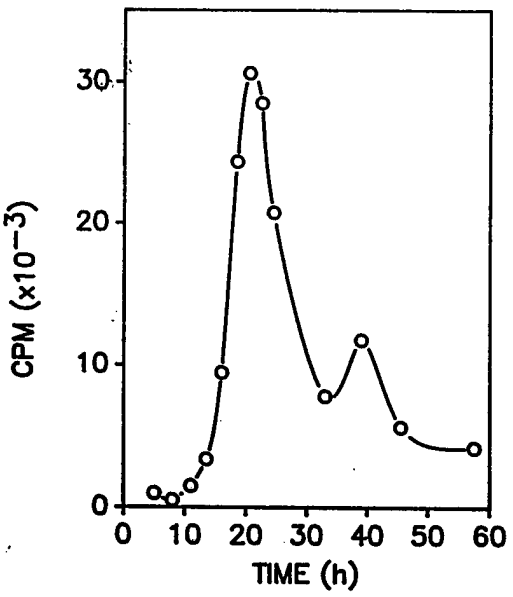


Figure 13. DNA Synthesis in Fibroblasts Incubated at 37°C. Cells were initially synchronized in G_0 at 37°C by serum starvation. At time 0, serum-supplemented media was added and cells were incubated continuously at 37°C. DNA synthesis was measured as [^{3}H]-thymidine incorporation at the indicated times.

If cells are held at 37°C for a few hours before transfer to 31°C, DNA synthesis begins progressively earlier as cells are held longer at 37°C (Figure 14, Panels B through E). If the cells are held at 37°C for 14 h, the peak of synthesis occurs at the same time as it does if they are maintained at 37°C throughout (Figure 14, Panel E).

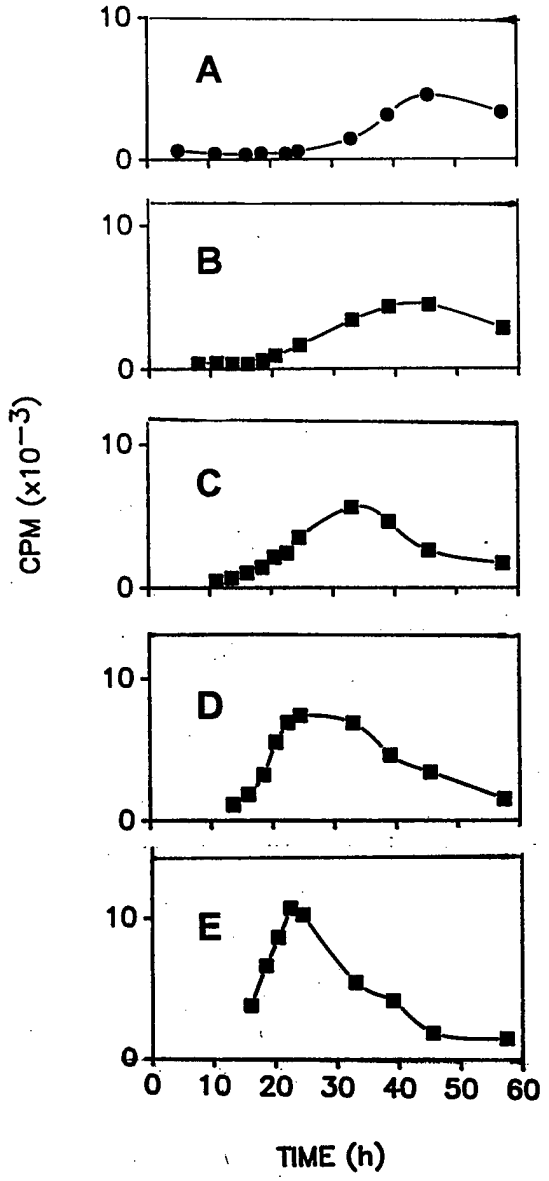


Figure 14. Delay in DNA Synthesis Caused by Mild Hypothermia. Cells were initially synchronized in G_0 at 37°C by serum starvation. At time 0, serum-supplemented media was added as in Figure 13 and cells were transferred to 31°C after varying lengths of time at 37°C. DNA synthesis was measured as [^{3}H]-thymidine incorporation at the indicated times. Panel A, immediate transfer to 31°C; Panel B, transfer after 5 h at 37°C; Panel C, transfer after 8 h at 37°C; Panel D, transfer after 11 h at 37°C; and Panel E, transfer after 14 h at 37°C.

The experiments shown in Figure 14 do not separate entrance into S phase from rate of synthesis once it begins. Accordingly, the experiments shown in Figures 15 and 16 were performed to examine the actual rate of DNA synthesis at 31°C. Cells were grown at 37°C for 7 h after plating and then exposed to aphidicolin (2.5 μg/ml) for 13 h to arrest them in S phase. After replacing the aphidicolin media with fresh media, cells were incubated at either 37°C or 31°C, and DNA synthesis was followed by ^{3}H-thymidine incorporation.

As shown in Figure 15, DNA synthesis began immediately after aphidicolin removal either at 37°C or 31°C, but the rate was much greater at 37°C. Thus, ^{3}H-thymidine incorporation was at a maximum after approximately 3 h at 37°C, but it took approximately 6 h for ^{3}H-thymidine incorporation to reach a maximum at 31°C.

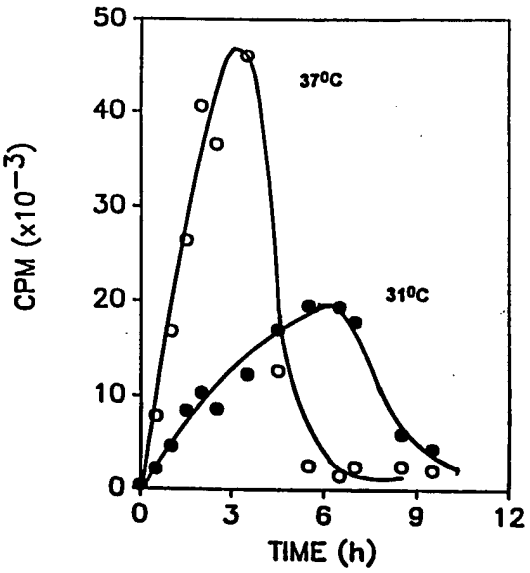


Figure 15. Effect of Hypothermia on DNA Synthesis in Cells Released from an Aphidicolin G_1/S Phase Block. Cells were incubated for 7 h at 37°C and then exposed to aphidicolin for 13 h at 37°C to block DNA synthesis. Upon removal of aphidicolin (time zero), cells were incubated at either 37°C (o) or 31°C (●). DNA synthesis was measured as [^{3}H]-thymidine incorporation at the indicated times following removal of aphidicolin.

Cell cycle analyses performed on these cells are shown in Figure 16 (next page). As shown in Panel A of that figure, cells were arrested at the very beginning of the S phase by aphidicolin. After release from the aphidicolin block, cells incubated at 37°C progressed rapidly through S phase (Panel B) and by 8.5 h are distributed between G_1 and G_2 (Panel C). By contrast, cells at 31°C had not progressed as far into the S phase at 3 h (Panel D), and some were still in S phase 8.5 h later although most were in G_2 (Panel E). These results are shown quantitatively in Table 1 on the next page: 3 h after release, approximately 70% of the cells are in S at both temperatures. However, after 8.5 h 37°C cells have already entered into the next cell cycle (71% in G_1) while 60% of the 31°C cells are still in G_2.

We conclude from this that mild hypothermia in addition to causing the G_1 arrest seen in Figure 12, also decreases the rate of DNA synthesis in S phase. The success of hypothermia in diminishing cytotoxicity will probably depend on delaying DNA synthesis sufficiently for repair to be complete.

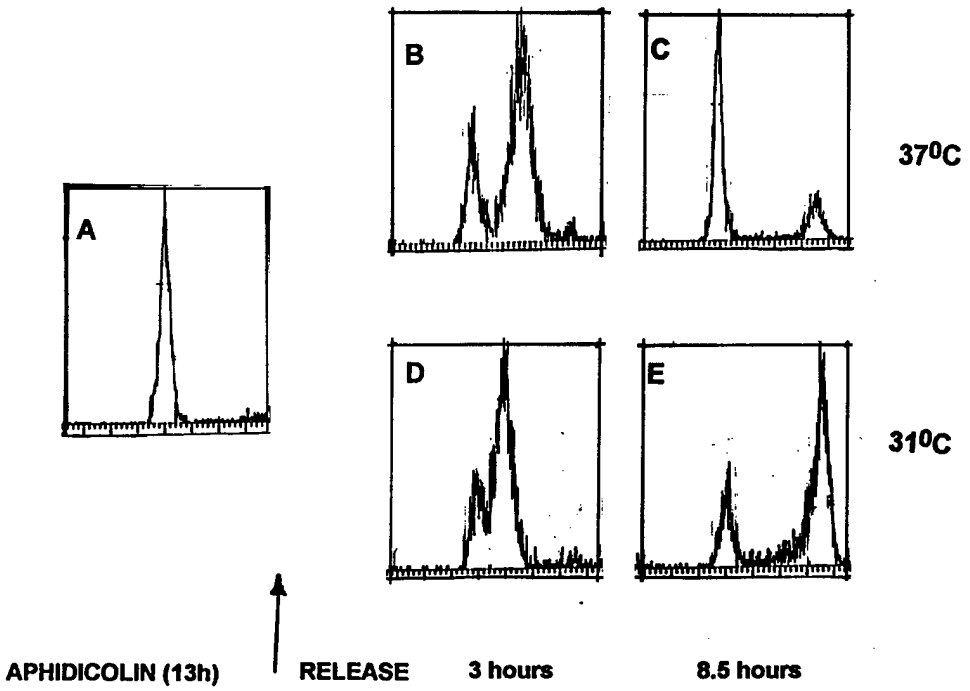


Figure 16. Effect of Hypothermia on Cell Cycle Progression in Cells Released from an Aphidicolin G_1/S Phase Block. Cells were exposed to aphidicolin for 13 h at 37°C to block DNA synthesis (Panel A). Upon removal of aphidicolin (time zero), cells were incubated at either 37°C (Panels B and C) or 31°C (Panels D and E). Cell cycle distribution was determined by FACS-analysis 3 h or 8.5 h after release from aphidicolin.

Table 1. Cell Cycle Distribution (Percent) Following Release from Aphidicolin Block

Time after release (h)	37°C			31°C		
	G_1	S	G_2	G_1	S	G_2
0	91	5	3			
3.0	23	72	3	25	70	3
8.5	71	6	21	20	17	60

Having established that hypothermia causes a reversible cell cycle arrest, we next questioned the nature of the signaling mechanism that causes this arrest. Cell cycle arrest in response to DNA damage is predominantly a manifestation of the p53-dependent transactivation of the cyclin-CDK inhibitor, p21. To examine the effects of hypothermia on cellular p53 and p21 protein levels, Western blot analyses were performed with whole cell extracts from AG01522 cells incubated at either 37°C or 28°C.

As shown in Figure 17, both p53 and p21 accumulated in cells incubated at 28°C. However, the time course for the accumulation of these two proteins was different. The highest level of p21 was found in cells after 7 hours of incubation at 28°C while p53 levels peaked after 12 hours. By the time the p53 level had reached its maximum, the p21 level had declined to control levels. Since p21 levels had fallen before the cells were released from G1 block, this suggests that although p21 may be needed to trigger arrest, high levels of p21 are not required for cells to remain in the arrested state.

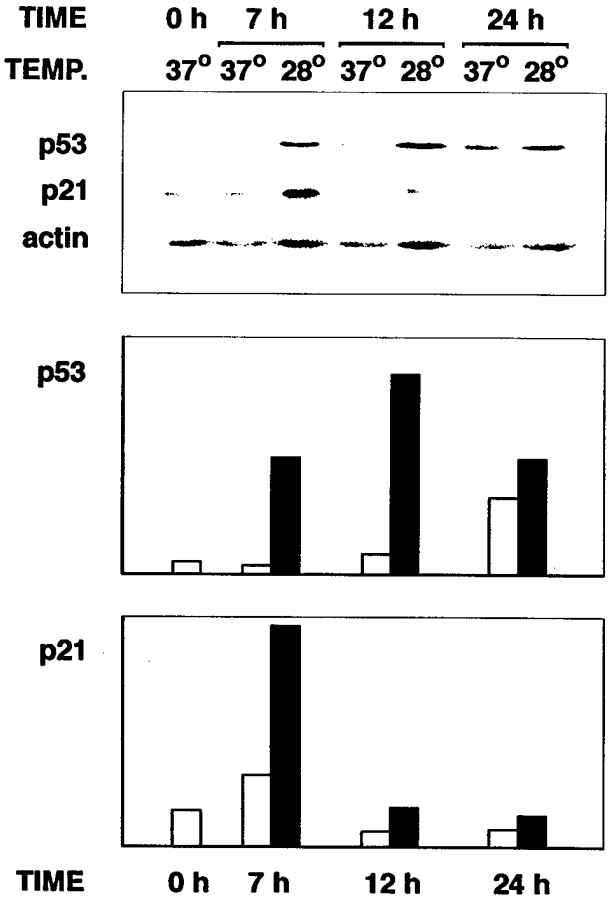


Figure 17. p53 and p21 levels in human AG01255 fibroblasts grown at 37°C or at 28°C. Cells were plated at a density of 2 x 10^4 cells/cm^2 and incubated at either 37°C or 28°C. At the indicated times, cells were lysed and protein (50 μg) was separated by PAGE on 10% SDS gels. Western blot-analyses (upper panel) were performed as described in the text. Levels of p53 (middle panel) and p21 (lower panel) were determined densitometrically relative to β-actin. Open bars, 37°C; solid bars, 28°C.

Since p53 levels rise in normal human fibroblasts in association with hypothermia-induced cell cycle arrest, the question arises as to whether p53 is actually required for the arrest to occur. If p53 is directly involved, cells that lack the gene for p53 should be unable to arrest when exposed to hypothermic conditions. We tested this by comparing cell cycle progression during hypothermia in isogenic mouse fibroblasts that are either wild type or null mutants for this tumor suppressor gene.

As shown in Figure 18, mouse fibroblasts that contain p53 underwent a cell cycle arrest when they were incubated at 28°C; cell cycle analysis showed no progression (top row, Figure 18). Mouse fibroblasts that lack the gene for p53, however, continued to progress through the cell cycle (bottom row, Figure 18). We conclude from this experiment that the presence of functional p53 is essential for hypothermia-induced cell cycle arrest.

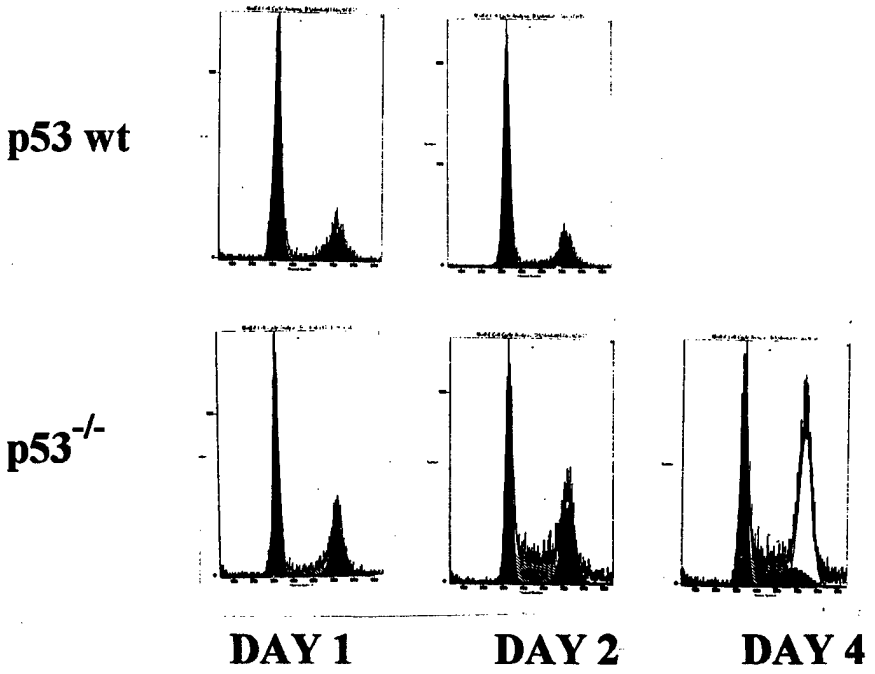


Figure 18. Effect of p53 on cell cycle progression in mouse embryo fibroblasts during hypothermia. Cell cycle analysis was performed on mouse embryo fibroblasts that were incubated at 28°C. Cells that contain p53 (top row) were arrested while cells that lack the gene for this protein (bottom row) continued to progress.

DISCUSSION

Formation of DNA-protein Cross-links

In recent years, major emphasis has been placed on transcriptional regulation as a process absolutely necessary for proper cellular function. It seems obvious that formation of DNA-protein cross-links involving regulatory protein elements would have very serious consequences, but the significance of these lesions cannot be determined without a method of measuring their occurrence.

The presence of a radiolabeled peak in HPLC profiles of ^{32}P-postlabeled nucleotides from DNA of cells exposed to SM (see Figure 3) offers an opportunity to measure these cross-links. However, it is necessary to synthesize the putative cross-link to prove that it is represented by the unknown radiolabeled peak.

Since only small quantities of the putative cross-link are needed, and its initial characterization depends on its having the correct HPLC retention time, we attempted to produce the required product following the scheme shown in Figure 2 in a "one pot" synthesis without isolating intermediate products. Two products with approximately the correct HPLC retention times were obtained (see Figure 4), but neither had the structure that would be expected for the residue of a DNA-protein cross-link.

In fact, the tentative structure identifications shown in Figure 5 indicate extensive rearrangement of products under the conditions chosen for these reactions. "Unknown I" is actually of some interest because it has the structure of the DNA-protein cross-link that is formed when cells are exposed to ethylene dibromide, a suspect carcinogen (50). Kim *et al.* (50) believe that their product is formed from an initial reaction of ethylene dibromide with glutathione and a subsequent reaction with DNA. Although it is interesting that we have arrived at the core of their guanine-glutathione cross-link in our reactions, we believe that the structure we have proposed in Figure 1 is what would be expected from reactions of SM with nucleoproteins. Consequently, we are currently pursuing our synthesis (Figure 2) in a stepwise manner using milder reaction conditions that should minimize rearrangements.

Repair of SM-induced DNA Modifications

Action of Glycosylases on SM-modified DNA

Cloned Human Alkylpurine Glycosylase

In view of the activity of bacterial 3-methyladenine DNA glycosylase II towards SM-induced DNA modifications (44), we anticipated that mammalian cells would have an enzyme with similar specificity. Before a definite conclusion can be drawn about the activity of a candidate enzyme, however, it must be purified to near homogeneity since a combination of other activities - in this case endonucleases and phosphatases - may give positive results that are, in fact, artifactual.

Consequently, we have enlisted the collaboration of our colleague, Professor Michael Volkert of the UMass Department of Microbiology and Molecular Genetics, to clone the glycosylase isolated by Samson *et al.* (49) with the addition of a histidine tag to facilitate purification. Very recently, we have obtained this enzyme in purified form and find that it has no discernible activity towards SM-modified DNA (Figure 6).

We are surprised at this result in view of the characterization of human glycosylase as having a broad substrate specificity (52). However, recent data indicate that mammalian cells have more than one glycosylase (24,25). The human glycosylase gene has been located on chromosome 16 and sequenced by Vickers *et al.* (52); this group of investigators compared the sequence that they obtained with those obtained by others who had cloned the gene (49,53,54), and found splicing differences in the N-terminal portion of the glycosylase. Since Roy *et al.* (55,56) believe that specificity of the glycosylase is determined

by this part of the molecule, it is possible that enzymes corresponding to these other sequences will have more activity towards SM-modified DNA. Histidine tags are being added to these other enzymes by our collaborator, Dr. Volkert, and we anticipate that purified enzyme from these genes will be available soon for testing.

Formamidopyrimidine-DNA Glycosylase (Fpg Protein)

Although the cloned human alkylpurine glycosylase has no demonstrated activity against SM-modified DNA, the Fpg protein is highly active against ring-opened 7-hydroxyethylthioethylguanine (r-o HETEG) as shown in Figures 7 and 8. Although the amount of r-o HETEG present in cellular DNA exposed to SM is not known, it is significant that the presence of this enzyme in cells exposed to a nitrogen-based alkylating agent are protected from its cytotoxic action (26). Presumably, the presence of the ring-opened form of HETEG interrupts DNA replication as does the presence of ring-opened 7-methylguanine (57). We conclude, therefore, that the Fpg protein probably does play a role in protecting cells from the cytotoxic action of SM.

Protection against SM Toxicity by the Nucleotide Excision Repair Pathway

Nucleotide excision repair is an alternative to glycosylase-initiated base excision repair for removing modified bases from DNA. Although this complex system is difficult to study *in vitro*, its importance in protecting cells from SM toxicity is clear from the experiments shown in Figure 9. Chinese Hamster Ovary (CHO) cells that lack this repair mechanism grow as well as those that are competent in this repair mechanism under normal conditions. However, deficient cells are very sensitive to SM toxicity as shown in the right hand panel of Figure 9. These results, besides establishing the importance of nucleotide excision repair in preventing cytotoxicity, also provide evidence that DNA modification is responsible for cytotoxicity.

Thus, to summarize our repair studies to date, it is clear that nucleotide excision repair plays an important role in protecting cells from SM toxicity. Base excision repair by the Fpg protein is probably also important, but the importance of repair by alkylpurine glycosylases is less certain. Proof of its importance will depend on demonstrating the *in vitro* action of other purified alkylpurine glycosylases, or the availability of cell lines deficient in this repair mechanism.

Studies of Hypothermia-induced Cell Cycle Arrest

The use of mild hypothermia appears to offer a relatively simple approach to ameliorating SM toxicity (33). Since we believe that the beneficial effects of hypothermia are exerted by slowing the cell cycle and allowing more time for DNA repair, we have studied the effects of hypothermia on cell cycle progression.

As a first step, we examined growth at different temperatures. As shown in Figure 10, normal human fibroblasts grow at a slower rate at 31°C than at 37°C and grow slowly, if at all, at 28°C. However, as shown in Figure 11, the arrest at 28°C is reversible and cells grown for as long as four days at 28°C recover when they are returned to 37°C.

Figure 12 shows cell cycle progression at these temperatures. Cells at 37°C go through a complete cell cycle in approximately one day and eventually arrest because of confluence. By contrast, cells at the lower temperatures proceed slowly through S and do not seem to enter the next cell cycle.

DNA synthesis was measured by ^{3}H-thymidine incorporation as shown in Figures 13 and 14. Cells synchronized in G_0 and grown at 37°C go through two waves of DNA synthesis approximately 20 h apart as shown in Figure 13. By contrast, entrance into S is greatly delayed at 31°C as shown in Panel A of Figure 14. If, however, cells are incubated for increasing lengths of time at 37°C before being transferred to 31°C, this delay is shortened. Finally (Panel E), if cells are held at 37°C for 14 h before transfer to 31°C, the peak rate of DNA synthesis is hardly delayed at all but synthesis continues over a longer period of time.

^{3}H-thymidine incorporation measurements, as performed in Figures 13 and 14, determine total DNA synthesis in the population of cells without separating effects from the number of cells entering S from those related to the rate of synthesis in each cell. Accordingly, we performed the experiments shown in Figures 15 and 16. Here, cells were arrested at the G_1/S boundary by introducing aphidicolin before the S phase would normally begin. On transfer to 31°C, the maximum rate of synthesis was again delayed as shown in Figure 15, but cell cycle analysis (Figure 16) indicates that S phase started promptly, but continued more slowly at 31°C.

We conclude that DNA synthesis occurs at a slower rate at 31°C and that this, combined with a G_1 arrest, would provide more time than would be available at 37°C for DNA repair. Maximum benefits would be derived from finding conditions under which DNA repair proceeds most rapidly in comparison with progression through the cell cycle.

Since the use of mild hypothermia appears to be a benign method of producing cell cycle arrest, an understanding of its mechanism might suggest other methods of inducing such an arrest. The experiments shown in Figure 17 indicate that the regulatory protein p53 is involved. Cells incubated at 28°C show a rapid increase in p53 levels in comparison with those incubated at 37°C.

To determine the significance of this rise in p53, we examined cell cycle progression in cells that were either wild type or null mutants for p53 as shown in Figure 18. Wild type mouse embryo fibroblasts arrested at 28°C just as do human fibroblasts, but as seen in the bottom panel of this Figure, cells lacking p53 continue to progress through the cell cycle. Thus, we would conclude that hypothermia-induced cell cycle arrest is indeed mediated by p53.

CONCLUSIONS

The data reported above support the following conclusions: 1. SM toxicity is initiated by DNA modification. 2. DNA repair mechanisms decrease SM toxicity. 3. Hypothermia slows cell cycle progression which allows more time for DNA repair before cellular division takes place. Hypothermia, therefore, may decrease toxicity after SM exposure.

Of the various DNA modifications caused by SM, DNA-protein cross-linking may be as important as DNA interstrand cross-linking in causing toxicity, but quantitative measurements of this phenomenon depend on the successful synthesis of a marker for use in our ^{32}P-postlabeling method. We believe that our synthetic route to this structure will be successful.

Nucleotide excision repair acts in mammalian cells to decrease SM toxicity as shown by the increased toxicity of SM to cells lacking this repair mechanism. *In vitro* studies show that the glycosylase, formamidopyrimidine-DNA glycosylase, acts on SM-modified DNA; coupled with literature data that show the presence of this enzyme decreases the cellular toxicity of aziridine, it seems probable that this repair mechanism also decreases SM toxicity. One purified mammalian alkylpurine glycosylase does not have any demonstrable activity towards SM-modified DNA, but other forms of this enzyme may be active in view of similar activity found in bacterial cells.

Mild hypothermia produces a generalized cell cycle arrest that is mediated by p53. It is likely that this allows more time for DNA repair to occur before the next cell division, thereby decreasing SM toxicity.

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APPENDIX

List of Publications Supported by this Contract:

1. Fpg protein releases a ring-opened N-7 guanine adduct from DNA that has been modified by sulfur mustard. Q. Li, J. Laval, and D. B. Ludlum. Carcinogenesis, *18*, 1035-1038 (1997).

2. FPG protein releases a ring-opened N-7 guanine adduct from DNA that has been modified by sulfur mustard. Q. Li, J. Laval, and D.B. Ludlum. Proc. Am. Assoc. for Cancer Res. *38*, 335 (1997).

3. Cellular responses to sulfur mustard alkylation. Z. Matijasevic, A. Stering, and D.B. Ludlum. Proc. Am. Assoc. for Cancer Res. *38*, 132 (1997).

4. Hypothermia causes a p53-medicated cell cycle arrest. Z. Matijasevic, J. E. Snyder, and D.B. Ludlum. In preparation.

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